

Hypoxia Signaling Cascade for Erythropoietin Production in Hepatocytes

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Erythropoietin (Epo) is produced in the kidney and liver in a hypoxia-inducible manner via the activation of hypoxia-inducible transcription factors (HIFs) to maintain oxygen homeostasis. Accelerating Epo production in hepatocytes is one plausible therapeutic strategy for treating anemia caused by kidney diseases. To elucidate the regulatory mechanisms of hepatic Epo production, we analyzed mouse lines harboring liver-specific deletions of genes encoding HIF-prolyl-hydroxylase isoforms (PHD1, PHD2, and PHD3) that mediate the inactivation of HIF1 α and HIF2 α under normal oxygen conditions. The loss of all PHD isoforms results in both polycythemia, which is caused by Epo overproduction, and fatty livers. We found that deleting any combination of two PHD isoforms induces polycythemia without steatosis complications, whereas the deletion of a single isoform induces no apparent phenotype. Polycythemia is prevented by the loss of either HIF2 α or the hepatocyte-specific *Epo* gene enhancer (*EpoHE*). Chromatin analyses show that the histones around *EpoHE* dissociate from the nucleosome structure after HIF2 α activation. HIF2 α also induces the expression of HIF3 α , which is involved in the attenuation of Epo production. These results demonstrate that the total amount of PHD activity is more important than the specific function of each isoform for hepatic *Epo* expression regulated by a PHD-HIF2 α -*EpoHE* cascade *in vivo*.

Under low-oxygen conditions (hypoxia), the number of circulating red blood cells increases to deliver oxygen efficiently into peripheral organs. The red blood cell mass is controlled by the erythroid growth factor erythropoietin (Epo), the majority of which is produced by the kidneys in a hypoxia-dependent manner (1). Therefore, inhabitants of high-altitude areas and patients suffering from chronic respiratory failure with chronic obstructive pulmonary disease often develop polycythemia (erythrocytosis) with elevated levels of Epo in their plasma (2, 3). Epo binds to its receptor (EpoR) on the surfaces of immature erythroid cells and stimulates signaling cascades for proliferation, differentiation, and antiapoptosis (4). *Epo* or *EpoR* gene-targeted mouse lines are embryonic lethal, with severe anemia around embryonic day 13, clearly indicating a requirement for Epo signaling in erythropoiesis (5).

Anemia often occurs in patients who suffer from kidney damage (6). We previously reported that renal Epo-producing (REP) cells that are in the interstitial spaces between renal tubules transform into myofibroblastic cells, which are closely associated with renal fibrosis under inflammatory conditions (7–11). Because the transformed REP cells lose their ability to produce Epo even under severely hypoxic conditions, pharmacologically inducing Epo production in the damaged kidneys of anemic patients is difficult. Although REP cells secrete most of the Epo in adult animals, hepatocytes are the primary Epo-producing cells in fetuses (12). The liver maintains its Epo-producing activity throughout adulthood; thus, *Epo* expression is detectable in the livers of anemic/hypoxic mice (8, 12). However, the level of hepatic Epo production is weak and insufficient to compensate for renal anemia (13). Therefore, pharmacological enhancement of hepatic Epo production is a reasonable strategy for treating anemic patients who have renal diseases (14, 15).

In hepatocytes, *Epo* transcription, which is the rate-limiting step of Epo production, is controlled by a *cis*-regulatory element that is proximally downstream of the *Epo* transcription end site (called *Epo* hepatic enhancer [*EpoHE*] in this study). *EpoHE* contains a binding sequence for hypoxia-inducible transcription factors (HIFs) (16). We previously demonstrated that *EpoHE* is necessary and sufficient for *Epo* expression in hepatocytes, whereas renal Epo production is independent of *EpoHE* (12, 13). Thus, while *Epo* expression is commonly induced by hypoxic stresses in REP cells and hepatocytes, these two cell types employ different mechanisms of *Epo* gene regulation.

HIFs are master regulators of hypoxia-inducible gene expression. Each HIF consists of an oxygen-responsive α subunit and a constitutively expressed nuclear β subunit (17). Mammalian ge-

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names contain three genes for HIF α isoforms. HIF1 α and HIF2 α have similar protein structures, and both exhibit strong transactivities under hypoxic conditions, whereas the function and regulatory mechanisms of HIF3 α remain controversial due to the existence of multiple HIF3 α splicing variants (18–20). Under normal oxygenation conditions (normoxia), HIF α subunits are consistently synthesized, and their specific proline residues are quickly hydroxylated by HIF-prolyl hydroxylase domain proteins (PHDs) (21, 22). Hydroxylated HIF α is recognized by von Hippel-Lindau protein (pVHL), which is a component of an E3 ubiquitin ligase complex, and degraded by the proteasome (23). Because hypoxia inhibits the enzymatic activities of PHDs, which use oxygen as a substrate, HIF heterodimers have increased activities as transcription factors under hypoxic conditions (24).

Hepatic Epo production is enhanced by the experimental targeting of the HIF pathway using genetically modified mice, small molecules, or small interfering RNA s (siRNAs) (25). For instance, liver-specific pVHL deficiency in mice results in polycythemia with the overexpression of the *Epo* gene in the liver (26). Because this phenotype depends on HIF2 α , it is thought that HIF2 α , rather than HIF1 α , regulates hypoxia-inducible *Epo* expression in hepatocytes (26). The contributions of the three PHD isoforms (PHD1, PHD2 and PHD3, which are encoded by the *Egln2*, *Egln1*, and *Egln3* genes, respectively) to hepatic *Epo* gene regulation have been investigated using gene-modified mice, and functional redundancy among the isoforms has been demonstrated (27–29). Although these strategies stimulate hepatic Epo production, the constitutive activation of HIFs induces steatosis and ectopic expression of cancer-related genes (25–29). Therefore, establishing a specific strategy for hepatic Epo induction to treat renal anemia is important, and fully understanding the molecular mechanism underlying *Epo* regulation will be necessary to develop such a strategy.

In this study, we analyzed gene-modified mouse lines lacking PHD isoforms, HIF2 α , and/or *EpoHE* specifically in the liver to elucidate the *in vivo* signaling pathway between hypoxia sensor PHDs and *cis*-regulatory elements in the hepatic *Epo* gene regulatory system. Our results demonstrate that the function of PHDs is gene dose dependent and that HIF2 α and *EpoHE* are necessary for hepatic Epo production. HIF2 α functions in both the removal of histones from the region around the *Epo* gene, which enhances *Epo* transcription, and the induction of HIF3 α expression to attenuate hepatic Epo production. Thus, we propose that a PHD-HIF2 α -*EpoHE* axis regulates hepatic Epo production by controlling HIF3 α expression and the chromatin structure of the *Epo* gene. Our results provide valuable information regarding therapeutic targets that enhance hepatic Epo production for renal-anemia patients.

MATERIALS AND METHODS

Mice. All mice were maintained at the Institute for Animal Experimentation, Tohoku University Graduate School of Medicine, under the Regulations for Animal Experiments and Related Activities of Tohoku University. A hypoxic box, in which the oxygen concentration was regulated by an oxygen controller (ProOx; Biospherix) with a nitrogen generator (Nilox; Sanyo Electronic Industries), was used to expose the mice to hypoxic conditions. For acclimation to severe hypoxia (6% O₂), the mice were exposed to 10% oxygen for 2 h before the oxygen concentration was reduced to 6%. Mouse lines carrying conditional knockout genes for PHDs (*Egln1*^{fl/fl}, *Egln2*^{fl/fl}, and *Egln3*^{fl/fl} genotypes) and HIF2 α (*Epas1*^{fl/fl} genotype) were used (30, 31). The *in vivo* function of the *Epo* gene hepatic enhancer (*EpoHE*) was investigated using mice with a deletion of *EpoHE*

(Δ EpoHE mice, *Epo* ^{Δ 3'E/ Δ 3'E} genotype) (9, 12). *AlbCre* transgenic mice were used to express Cre recombinase exclusively in mature hepatocytes (32). The *Epas1*^{fl/fl} and *AlbCre* mice were purchased from the Jackson Laboratory. Genotyping of genomic DNA from tails was performed by PCR using the primers listed in Table 1.

Reverse transcription and PCR. Total RNA samples from mouse organs were extracted using Isogen (Nippon Gene). cDNA was synthesized using a SuperScript III system (Invitrogen). To detect both normal and knockout transcripts derived from genes for PHDs simultaneously, cDNAs were amplified with primer pairs that annealed to sequences flanking the deleted exons (Table 1), and the PCR products were subjected to gel electrophoresis, followed by ethidium bromide staining (30). PCR primers that annealed to sequences in exons 3 and 4 of the *Hif3a* gene were used (Table 1) to detect both IPAS- and HIF3 α -specific splicing variants of the *Hif3a* gene by agarose gel electrophoresis because the IPAS mRNA contains 56-bp sequences from the IPAS-specific exon (exon 4a) that is between exons 3 and 4 (33). Quantitative PCR (qPCR) was performed on cDNA with the primers listed in Table 1, using FastStart SYBR green master mix with a LightCycler 96 system (Roche) or Power SYBR green master mix with a StepOne real-time PCR system (Life Technologies). The expression levels of β -actin mRNA were used as internal controls for the PCR experiments.

Blood analysis. Peripheral blood (0.2 to 0.3 ml) was collected from the mouse submandibular vein into a 1.5-ml tube containing 5 μ l of 0.5 M EDTA. After the blood samples were centrifuged, plasma Epo concentrations were measured using a mouse Epo enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems). To induce anemia by bleeding, 0.3 ml of peripheral blood was withdrawn from the mouse submandibular vein once per day for 3 days, and then the recovery from the anemia was investigated by measuring hematocrit levels in the retro-orbital venous plexus using heparinized microtubes (Drummond). The glucose concentration in blood from tail tips was directly measured using an animal glucometer (LAB Gluco; ForaCare).

Flow cytometry. Mononucleated cells from the spleen and bone marrow were prepared by isopycnic centrifugation with Histopaque-1083 (Sigma) and were stained with fluorescein isothiocyanate (FITC)-conjugated Ter119 (116215; BioLegend) and phycoerythrin (PE)-conjugated CD71 (113808; BioLegend) antibodies. After washing, the cells were sorted using a FACS Jazz flow cytometer (Becton Dickinson).

Immunoblotting. Nuclear extracts were obtained from mouse livers using sucrose buffer (0.25 M sucrose in 10 mM HEPES-KOH buffer) and resuspended in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-Cl, 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, and 1.0% NP-40) supplemented with inhibitors for proteinase (Roche) and proteasome (Proteintech). Whole-cell extracts were directly obtained with RIPA buffer. The cell extracts were incubated with tobacco etch virus (TEV) protease (Promega) at 4°C overnight to remove the HaloTag from HaloTag-HIF3 α fusion proteins. Proteins (25 μ g) were transferred to nitrocellulose membranes (Bio-Rad) after electrophoresis using 6% or 8% polyacrylamide gels, and the membranes were incubated with primary antibodies against HIF1 α (PAB12138; Abnova), HIF2 α (C150132; LSBio), HIF3 α (HPA041141; Sigma), green fluorescent protein (GFP) (598; MBL), Nup62 (ab96134; Abcam), α -tubulin (T5168; Sigma) or β -tubulin (PA1-21153; Thermo). Horseradish peroxidase-conjugated secondary antibodies against mouse (01803-44; Nacalai Tesque), rabbit (P0448; Dako) or goat (sc-2020; Santa Cruz) immunoglobulins were used with chemiluminescent detection reagents (GE Healthcare) to detect signals with a Chemi-Doc imaging system (Bio-Rad).

Glycogen assays. To measure the liver glycogen concentration, boiled supernatants from liver homogenates (2.0 mg tissue/ml water) were digested with glucoamylase for 30 min at room temperature. Samples were analyzed using the fluorometric assay protocol in the glycogen assay kit (BioVision). To detect glycogen in liver sections, periodic acid-Schiff (PAS) staining was conducted on paraffin-embedded sections using a PAS staining kit (Muto).

TABLE 1 Sequences of the primers used in this study

Purpose and target	Primer 1 (5'–3')	Primer 2 (5'–3')
Genotyping		
<i>Egln1</i> (PHD2)	CAAATGGAGATGGAAGATGC	TCAACTCGAGCTGGAACCC
<i>Egln2</i> (PHD1)	TGGGCGCTGCATCACCTGTATCT	ACTGGTGAAGCCTGTAGCCTGTC
<i>Egln3</i> (PHD3)	ATGGCCGCTGTATCACCTGTAT	CCACGTAACTCTAGAGCCACTGA
<i>AlbCre</i>	ACGTTACCCGGCATCAACGT	CTGCATTACCCGGTCGATGCA
<i>Epas1</i> (HIF2 α)	CAGGCAGCAGGCAGTATGCCTGGCTAATTCCAGTT	CTTCTTCCATCATCTGGGATCTGGGACT
<i>EpoHE</i>	CAGGCTCCATTCAAGGC	CCTGCAGTGGACTTTGAAGGC
RT-PCR with gel electrophoresis		
PHD1	ACCGCGCAGCATTCGTG	GGGGCTGGCCATTAGGTAGGTGTA
PHD2	GCGGGAAGCTGGGCAACTAC	CAACCCCTACACCTTTCTCACC
PHD3	CTGCGTGCTGGAGCGAGTCAA	TCATGTGGATTCTGCGGTCTG
IPAS/HIF3 α	GAGGGTTTCGTATGGTACT	TCTTGAAGTTCCTCTTGGTC
RT-qPCR with SYBR green reagents for mouse (m) or human (h) mRNA expression		
β -Actin (m, h)	GACAGGATGCAGAAGGAGAT	TTGCTGATCCACATCTGCTG
PHD1 (m)	ATGGCTCACGTGGACGCAGTAA	CATTGCCTGGATAACACGCCAC
PHD2 (m)	TAAACGGCCGAACGAAAGC	GGGTATCAACGTGACGGACA
PHD3 (m)	CTATGTCAAGGAGCGGTCCAA	GTCCACATGGCGAACATAACC
Epo (m)	CATCTGCGACAGTCGAGTTCTG	CACAACCCATCGTGACATTTTC
EpoR (m)	GGGTCCGAAGAAGCTTCTGTG	ATGACTTTCTGTGACTCACCTC
Bnip3 (m)	GTTACCCACGAACCCACTTT	GTGGACAGCAAGGCGAGAAT
Vegfa (m)	CTGCTGTAACGATGAAGCCCTG	GCTGTAGGAAGCTCATCTCTCC
Fasn (m)	GGCTGCTGTTGGAAGTCAGC	AGTGTTCGTTCTCGGAGTG
Scd1 (m)	TTCTTGCGATACACTCTGGTGC	CGGGATTGAATGTTCTTGTCTG
HIF1 α (m)	CCTGCACTGAATCAAGAGGTTGC	CCATCAGAAGGACTTGCTGGCT
HIF2 α (m)	CAATGACAGCTGACAAGGAG	CATAGAAGACCTCCGTCCTCC
HIF3 α (m)	AAGACGCCCTGACCCCGAGG	CCCTCTGCTGGTGAGCGTGC
Gpi (m)	CCCTCTTTATAATCGCCTCCA	GAAACCACTCCTTTGCTGTCTC
Ldha (m)	GGCACTGACGCAGACAAG	TGATCACCTCGTAGGCACTG
Hepcidin (m)	TCTTCTGCATTGGTATCGCA	GAGCAGCACCACCTATCTCC
CA9 (h)	CCTTTGCCAGAGTTGACGAG	GACAGCAACTGCTCATAGGC
PGK1 (h)	CTAACAAGCTGACGCTGGAC	CTGGTTGTTTGTATCTGGTTG
EPO (h)	TCATCTGTGACAGCCGAGTC	CAAGCTGCAGTGTTTCAAGC
qPCR with SYBR green reagents for chromatin analysis		
CA9 promoter	TCTGTGAGTCAGCCTGCTCC	TCCCAGCACACGGTGTGTAC
PGK1 promoter	CCCTAAGTCGGGAAGGTTCC	CTGTCCGTCTGCGAGGGTAC
EPO promoter	CTCAACCCAGGCGTCTCG	GGGGCTGTTATCTGCATGTG
EPO intron1	CTGTTTGAGCGGGGATTTAGC	TTCCGGGGTCTTGACAAGT
EPO HE	AACCTCCAAATCCCCTGGCTC	CTGTGTGAGACAGCAGTAG

Lipid droplet staining. Frozen sections (10- μ m thickness) of mouse livers that had been fixed in 4% formaldehyde for 4 h were incubated with Nile red solution (100 ng/ml; Wako) and Hoechst 33342 (1.0 μ g/ml; Calbiochem) for 5 min (34). After the sections had been washed with PBS, fluorescence signals were detected using the pseudocolor detection system of a BZ9000 microscope (Keyence).

Cell culture and reporter analysis. Hep3B cells that were derived from a human hepatoma were cultured in high-glucose Dulbecco's modified Eagle medium (DMEM) (Nacalai Tesque) supplemented with 10% fetal bovine serum (Sigma) (35). For hypoxic cell sampling, the cells were incubated and treated in a Sci-tive hypoxia workstation (Ruskin), in which the air was composed of 1% O₂, 5% CO₂, and 94% N₂.

The plasmid pT81/HRE-luc, which expresses firefly luciferase under the control of HIF-binding sequences from *EpoHE*, was used as a reporter for *EpoHE* activity, and pCMX-HIF2 α CA was used to overexpress Flag-tagged mouse HIF2 α bearing constitutively activated mutations in proline residues (P405A and P530A) (36). We used the pFN21AB6219 plasmid (Kazusa Institute) to express human HIF3 α linked to a HaloTag peptide (Promega).

For transient-transfection assays, the cells were cultured to 70% confluence in 12-well plates and transfected with pT81/HRE-luc (300 ng/well), pCMX-HIF2 α CA (300 ng/well), and/or pFN21AB6219 (300 or 600 ng/well) using Lipofectamine 2000 (Life Technologies). For internal transfection efficiency controls, 100 ng/well of the reference plasmids pEGFP-N1 (TaKaRa) and pRL-EF (37) were added to all transfection mixtures. The cells were harvested at 48 h after transfection, and the activity of firefly luciferase was measured using a dual luciferase reporter assay system (Promega) after normalization with the activity of *Renilla* luciferase, which was expressed under the *EEF1A1* gene promoter from pRL-EF (37). The cells were observed under a BZ9000 microscope after supplementation of the culture medium with tetramethylrhodamine (TMR)-conjugated HaloTag direct ligand (Promega) to detect HaloTag-HIF3 α expression in living cells.

Chemical compounds. FG4592 (Selleck Chemicals) and acriflavine hydrochloride (Sigma) were dissolved in dimethyl sulfoxide (DMSO) (Nacalai Tesque). HIF2 antagonist 2 {N-(3-chloro-5-fluorophenyl)-4-nitrobenzo[c]1,2,5-oxadiazol-5-amine; CAS no. 1422955-31-4} was synthe-

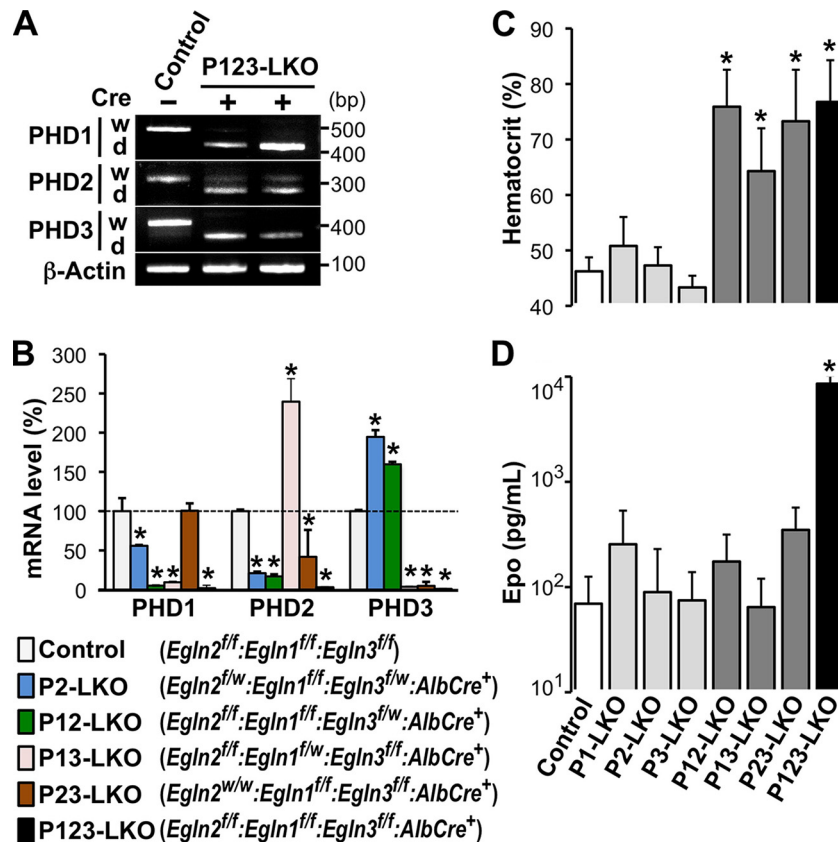


FIG 1 Liver-specific knockout of multiple PHD isoforms causes polycythemia. (A) RT-PCR was performed on livers from P123-LKO (*Egln2^{fl/fl}:Egln1^{fl/fl}:Egln3^{fl/fl}:AlbCre⁺*) genotype or control (Cre⁻ *Egln2^{fl/fl}:Egln1^{fl/fl}:Egln3^{fl/fl}* genotype) mice. Then, the PCR products were subjected to agarose gel electrophoresis to detect the expression of longer and shorter transcripts from the normal (w) and targeted (d [deletion of the exon encoding the catalytic domain of each PHD isoform]) alleles, respectively. β-Actin was used as an internal control. (B) Average expression levels of PHD isoforms in the livers of the indicated genotype mice were measured by RT-qPCR. The values are averages for 3 to 5 mice in each group, with error bars indicating standard deviations (SD). *, *P* < 0.01 compared to the expression levels in the control mice, which are set at 100%. (C and D) Average hematocrit (C) and Epo concentration (D) in peripheral blood samples from PHD mutant mice at 8 to 19 weeks after birth. The values are averages for 4 to 12 mice in each group, with error bars indicating SD. *, *P* < 0.01 compared to the levels in the control mice.

sized from 3-chloro-5-fluoroaniline (Sigma-Aldrich) and 5-chloro-4-nitrobenzo[c]1,2,5-oxadiazole (Namiki) in accordance with a previously published protocol (38) and resuspended in DMSO after purification.

Chromatin analysis. Nuclear extracts of Hep3B cells were partially digested with nucleases from an EpiQ chromatin analysis kit (Bio-Rad) to detect the chromatin states of HIF target genes. Using purified genomic DNA from nuclease-digested and undigested samples, qPCR was conducted with the primers listed in Table 1, and the abundance of PCR target sites within the two samples was compared. Because chromatin regions with nucleosome structures (genomic DNA masked by histones) are resistant to nuclease digestion (accessibility), a similar number of target sites were observed in the histone-rich regions in the nuclease-digested and undigested samples (100% protection from nuclease digestion). Nucleosome-free chromatin regions show low levels of protection (39).

Statistics. The data are presented as means ± standard deviations (SD). *P* values were calculated using two-tailed, unpaired Student's *t* tests.

RESULTS

Polycythemia is induced by the loss of two PHD isoforms in the liver. Mice harboring conditional knockout (floxed) genes for PHD1, PHD2, and PHD3 were crossed with *AlbCre* transgenic mice expressing Cre recombinase in hepatocytes to elucidate the roles of the PHD isoforms in hepatic Epo production (30, 32). Because *loxP* sequences were inserted at both ends of the exon that

encodes the catalytic domain for each PHD in the floxed alleles (f), the PHD transcripts that were produced following Cre-mediated liver-specific knockout (LKO) were shorter than those generated by the wild-type and floxed alleles (30). The shorter transcripts were predominantly detected in the livers of triple-knockout mice (*Egln2^{fl/fl}:Egln3^{fl/fl}:AlbCre⁺* genotype; called P123-LKO mice), whereas the control mice, which did not carry the Cre transgene (*Egln2^{fl/fl}:Egln1^{fl/fl}:Egln3^{fl/fl}* genotype), expressed only transcripts of normal length (Fig. 1A). These data indicated that the expression of functional PHD isoforms was almost eliminated by *AlbCre* transgene expression in the hepatocytes of P123-LKO mice. Additionally, the levels of mRNA expression for each PHD isoform in the livers of PHD mutant mice were quantitatively measured by reverse transcription-quantitative PCR (RT-qPCR), and the data confirmed the high efficiency of *AlbCre*-mediated liver-specific gene targeting (Fig. 1B). Interestingly, PHD2 mRNA expression in PHD1/PHD3 double-knockout (P13-LKO) livers was higher than that in the control mice. In addition, PHD3 mRNA expression was increased by the loss of the genes for the other PHD isoforms in livers (Fig. 1B). These data suggest that the expression of the genes for PHD2 and PHD3 is induced by HIFs (40, 41).

Next, we measured the hematocrit levels (red blood cell mass)

in the peripheral blood samples of adult mice (11 to 39 weeks of age) lacking a single PHD isoform in the liver (P1-LKO, P2-LKO, and P3-LKO mice). These mutant mice had no apparent abnormalities, with normal hematocrit levels compared with control mice lacking the Cre transgene (Fig. 1C). A double knockout of PHD isoforms in any combination (P12-LKO, P13-LKO, and P23-LKO mice) resulted in polycythemia, with hematocrit levels ranging from 65% to 85% (Fig. 1C). Mice lacking all three PHD isoforms in the liver (P123-LKO mice) exhibited polycythemia with a severity similar to that observed in the double-knockout mice (Fig. 1C). These data demonstrate that the inhibition of two PHD isoforms in the liver is necessary and sufficient for erythropoietic induction and that the total dose of PHD isoforms in the liver seems to be more important for PHD suppressive activity in erythropoiesis than the specific function of each isoform.

We observed that the Epo concentration in the plasma of P123-LKO mice was significantly higher than that in control mice (Fig. 1D). This result is consistent with the hematocrit data from polycythemic P123-LKO mice. However, plasma Epo levels in double-knockout mice were normal, although the mice suffered from polycythemia (Fig. 1C and D).

Loss of two PHD isoforms induces *Epo* expression in the liver. Although the liver-specific double knockout of PHD isoforms was thought to cause polycythemia through Epo oversecretion from the liver, the plasma Epo concentrations in these double-knockout mice were within the normal range (Fig. 1D). Thus, we measured the level of *Epo* expression in the livers of the PHD mutant mice. *Epo* expression was increased 10- to 20-fold by the loss of 2 PHD isoforms in the liver (Fig. 2A), while single PHD isoform knockout did not alter the *Epo* mRNA levels (28, 29). In the triple-knockout mice, the hepatic *Epo* mRNA level was dramatically (approximately 200-fold) higher than levels in control and double-knockout mice (Fig. 2A). These data demonstrated that PHD isoforms functionally compensate for each other in hepatic *Epo* production and that any single isoform can partially suppress hepatic *Epo* expression under normal conditions.

Next, we predicted that most of the circulating Epo in the double-knockout mice was consumed through the internalization of Epo-EpoR complexes by an expanded population of erythroblastic cells in the spleen after erythropoiesis stimulation (42). As expected, the spleens were significantly enlarged in the PHD double-knockout mice and in the triple-knockout mice (Fig. 2B and C). Splenic EpoR mRNA levels, which were normalized to β -actin mRNA levels, were extremely high in the double-knockout mice compared with control mice (Fig. 2D). EpoR expression was also slightly induced in the bone marrow of liver-specific PHD double-knockout mice (Fig. 2D). Flow cytometry of splenic mononucleated cells demonstrated that the Ter119⁺ CD71⁺ erythroblastic cell fraction of the double-knockout mice was approximately 10-fold higher than that in control mice (Fig. 2E) (13, 43). These data suggest that the excess Epo produced by the PHD-deficient livers is absorbed by the expanded erythroblastic cells in spleens. This hypothesis may explain why plasma Epo levels are normal in polycythemic mice lacking two PHD isoforms in their livers.

To test this hypothesis, the hematocrit levels in double-knockout mice were reduced to normal by phlebotomy, and then the postphlebotomy hematocrit levels were evaluated over time. As expected, the mice reverted to their original polycythemic phenotype within 3 weeks postphlebotomy (Fig. 2F), and their plasma Epo levels fell to within the normal range throughout the obser-

vation period. These findings suggest that these double-knockout mice constitutively secrete excess Epo from the liver but that most of the Epo is internalized by erythroblasts after initiating the signaling cascade that induces erythropoiesis. Because a large difference in Epo mRNA levels between the double- and triple-knockout livers exists (Fig. 2A), the rate of Epo production in P123-LKO livers likely surpasses the rate of internalization.

Liver-specific loss of all PHD isoforms causes severe steatosis and growth retardation. Immunoblotting of liver nuclear extracts demonstrated that protein levels of both HIF1 α and HIF2 α were increased by triple knockout of the PHD isoforms (Fig. 3A), while HIF proteins were undetectable in livers of the double-knockout, single-knockout, and control mice. These data are consistent with previous reports that HIF protein expression is very low or undetectable in PHD double-knockout livers compared with triple-knockout livers (28, 29). Therefore, it is possible that the low-level accumulation of HIF1 α and HIF2 α is sufficient to induce hepatic *Epo* gene expression and polycythemia in PHD double-knockout mice. Loss of all 3 PHD isoforms in hepatocytes results in a drastic induction of *Epo* expression via the hyperactivation of HIFs. Thus, PHD isoforms compensate for each other in the suppression of HIFs in hepatocytes under normal conditions, and no apparent abnormality is caused by loss of one of the three isoforms. The presence of a single functional PHD isoform is insufficient for the oxygen-dependent inactivation of HIFs. HIF1 α and/or HIF2 α escapes degradation in the hepatocytes and, although present at undetectable levels, induces *Epo* overexpression in the livers of double-knockout mice with polycythemia.

Because we showed that *Epo* expression was induced by HIF activation in mice lacking more than two PHD isoforms in the liver, the expression of other HIF target genes was analyzed in the livers of P23-LKO and P123-LKO mice. The results demonstrated that the expression of *Bnip3* and *Vegfa* mRNA was slightly increased in both P23-LKO and P123-LKO mouse livers compared to control mouse livers (Fig. 3B). These data demonstrated that PHD inactivation causes not only *Epo* production but also the global induction of HIF target genes, some of which promote mitochondrial autophagy (*Bnip3*) and angiogenesis (*Vegfa*).

The livers of P123-LKO mice at 7 weeks of age were large and fatty compared to the livers of mice in other groups. Nile red staining of lipid droplets in the liver sections clearly showed that P123-LKO mice suffered from severe steatosis (Fig. 3C). The double-knockout mice exhibited no lipid droplet accumulation in the liver (Fig. 3C). These data support previous reports showing that the liver-specific activation of the HIF pathway via the genetic inactivation of pVHL causes steatosis (44, 45). The expression of the genes for *Fasn* and *Scd1*, both of which are involved in fatty acid synthesis, was strongly suppressed in P123-LKO livers (Fig. 3B). This finding suggests that mitochondrial function is inhibited by HIF activation via *Bnip3*-mediated mitophagy (46) and that the accumulation of lipids due to decreased fatty acid usage in mitochondria may negatively regulate the expression of fatty acid synthesis genes. Consistent with a previous report that demonstrated increased glycogen concentrations in response to pVHL deficiency in liver (47), the loss of more than 2 PHD isoforms resulted in increased hepatic glycogen deposition (arrows in Fig. 3D). Indeed, the glycogen concentration in P23-LKO mouse livers was significantly higher than that in control mouse livers (Fig. 3E).

P123-LKO mice exhibited growth retardation (Fig. 3F) and a shortened life span of 14 weeks (Fig. 3G). These observations in-

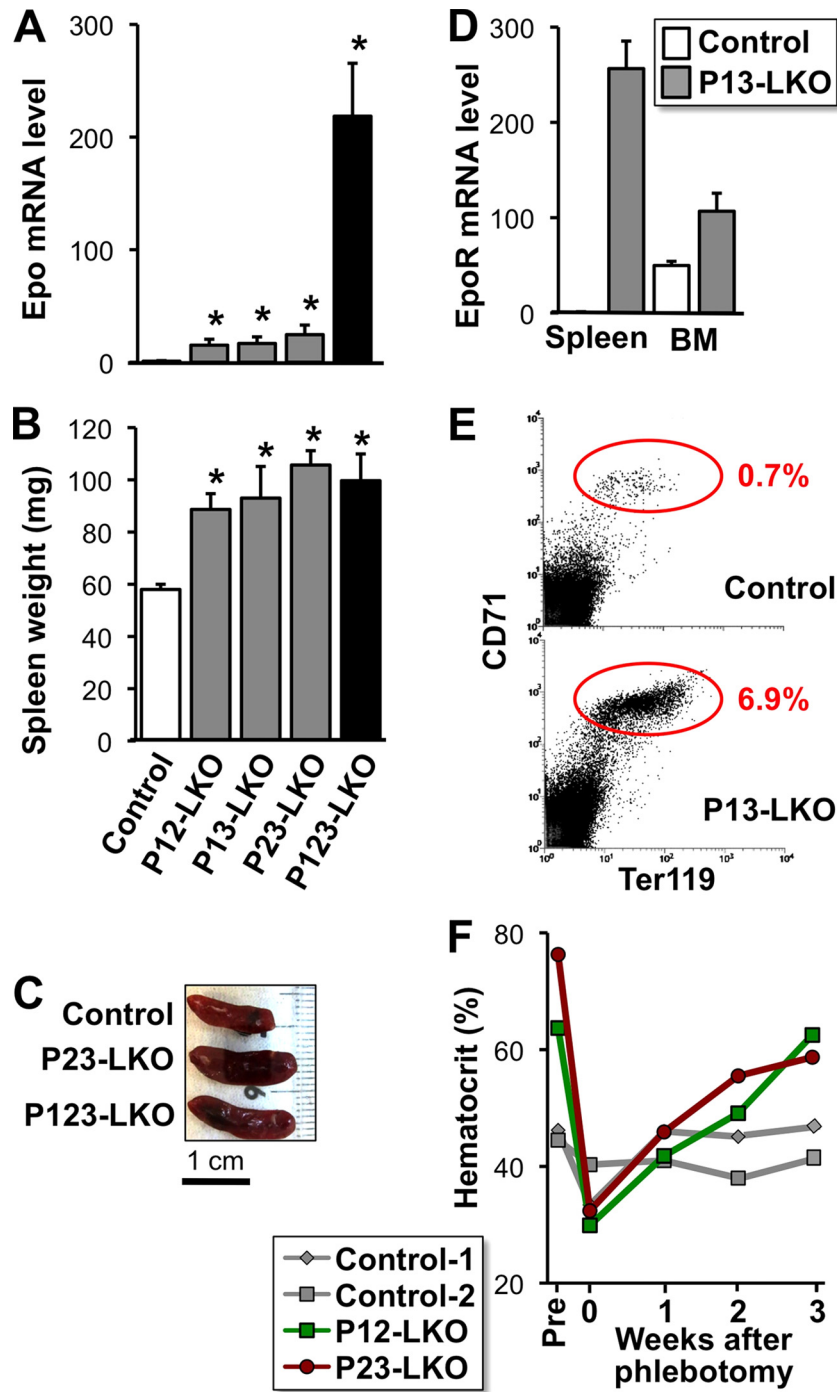


FIG 2 Overexpression of the *Epo* gene in livers lacking PHD isoforms. (A) Epo mRNA expression levels in the livers of PHD mutant mice at 8 to 12 weeks of age. The average expression level of control mice was set as 1.0 after normalization to β -actin mRNA levels. The data are means \pm SD ($n = 3$ for each group). *, $P < 0.01$ compared to the levels in the control mice. (B) Splenic hypertrophy in liver-specific PHD-deficient mice. Wet weights of spleens from the PHD mutant mice (7 to 9 weeks of age) were measured. The values are means and SD ($n = 3$ for each group). *, $P < 0.01$ compared to the weights of control mouse spleens. (C) Spleens from the PHD mutant mice were enlarged at 8 weeks of age. (D) EpoR mRNA levels in the spleens and bone marrow of control and P13-LKO mice. The average expression level in control mouse spleens was set as 1.0 after normalization to β -actin mRNA levels. The data are means and SD ($n = 3$ for each group). (E) Representative data from flow cytometry with CD71 and Ter119 antibodies in mononucleated cells from spleens of control and P13-LKO mice. The percentages of cells found within the circles are shown. (F) Changes in the hematocrit levels of the PHD mutant mice before (Pre) and after phlebotomy. The hematocrit levels in mice at 12 weeks of age were decreased to approximately 30% by phlebotomy. Note that the hematocrit levels of PHD mutant mice returned to polycythemic levels within 3 weeks after phlebotomy.

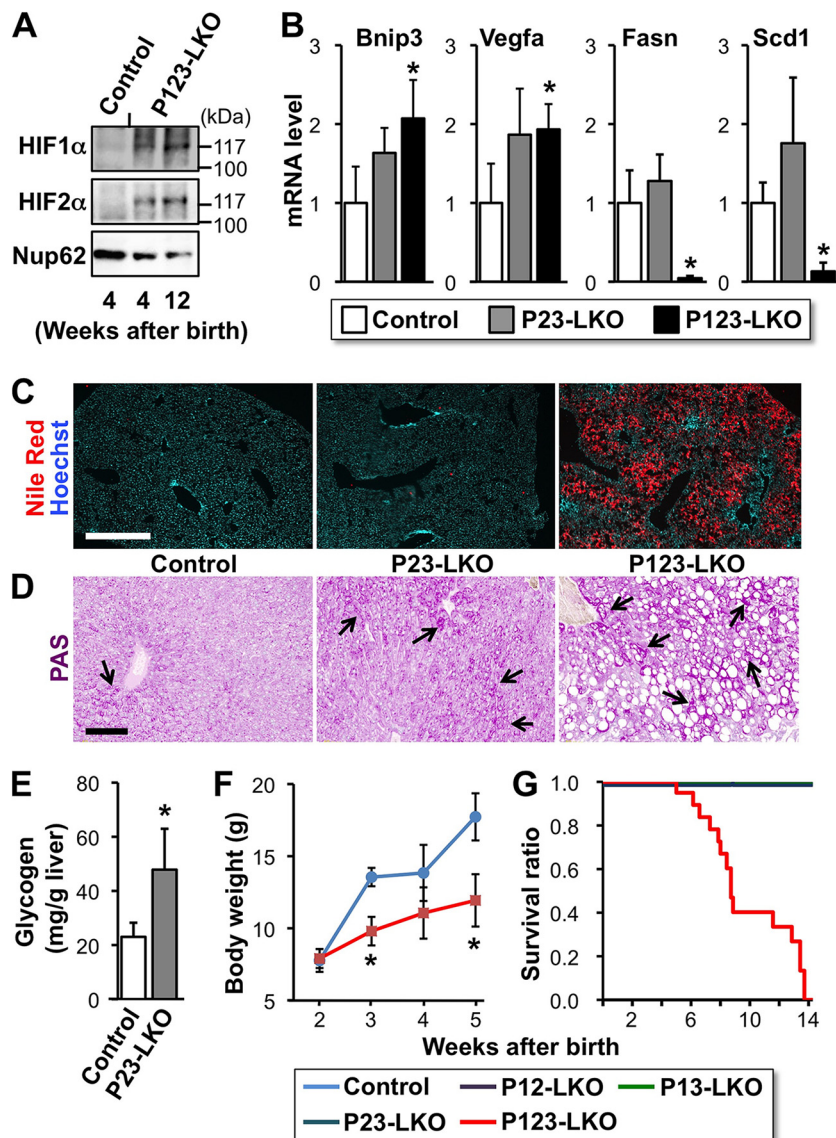


FIG 3 Disruption of all three PHD isoforms in the liver causes steatosis and growth retardation. (A) Immunoblots for HIF1α and HIF2α in nuclear extracts from the livers of P123-LKO mice at the indicated ages. Nup62 was used as an internal control. (B) Expression levels of Bnip3, Vegfa, Fasn, and Scd1 mRNAs in the livers of PHD mutant mice were analyzed by RT-qPCR. β-Actin was used as an internal control. The expression levels of control mice were set as 1.0. The data are means and SD ($n = 3$ for each group). (C) Lipid droplets in the livers of the PHD mutant mice at 7 weeks of age were stained with Nile red (red). Hoechst 33342 was used for nuclear staining (blue). (D) Glycogen deposition in the livers of PHD mutant mice at 8 weeks of age was detected by PAS staining (arrows). Bars, 500 μm (C) and 100 μm (D). (E) The glycogen concentration was measured in the livers of the control and P23-LKO mice at 8 weeks of age. The data are means and SD ($n = 4$ for each group). (F) Changes in body weight between 2 and 5 weeks after birth in P123-LKO and control mice. Error bars indicate SD ($n = 3$ for each group). (G) Kaplan-Meier survival analysis of the indicated genotype mice ($n = 20$ for each group). More than half of the P123-LKO mice died by 10 weeks after birth. *, $P < 0.01$ compared with the control mice (B, E, and F).

indicate that the universal knockout of all PHD isoforms in livers dramatically increases HIF activity, which causes severe steatosis and lethality. Because the inactivation of two PHD isoforms was sufficient to induce polycythemia without steatosis and lethality, we propose that mild inhibition of PHDs leads to the activation of hepatic Epo production without severe side effects.

HIF2α is a major regulator of hypoxia-inducible *Epo* gene expression in the liver. PHD mutant mice with the *AlbCre* transgene were mated with HIF2α conditional knockout mice to investigate whether HIF1α or HIF2α activates the *Epo* gene in PHD-deficient hepatocytes (31). The polycythemia exhibited by the

double-knockout mice was completely rescued by the loss of the HIF2α gene (*Epas1*) in hepatocytes (P12H2-LKO, P13H2-LKO, and P23H2-LKO mice in Fig. 4A). The hematocrit levels of HIF2α-deficient P123-LKO (P123H2-LKO) mice were lower than those of P123-LKO mice (Fig. 1C and 4A). Similar to the results of deleting the PHD isoforms in hepatocytes, pVHL deficiency causes Epo overexpression and polycythemia in mice, and this phenotype is blunted by the loss of HIF2α but not of HIF1α (26). Taken together, these data demonstrate that HIF2α, but not HIF1α, predominantly activates the hepatic *Epo* gene under hypoxic conditions by signaling through PHDs. Because the lethality

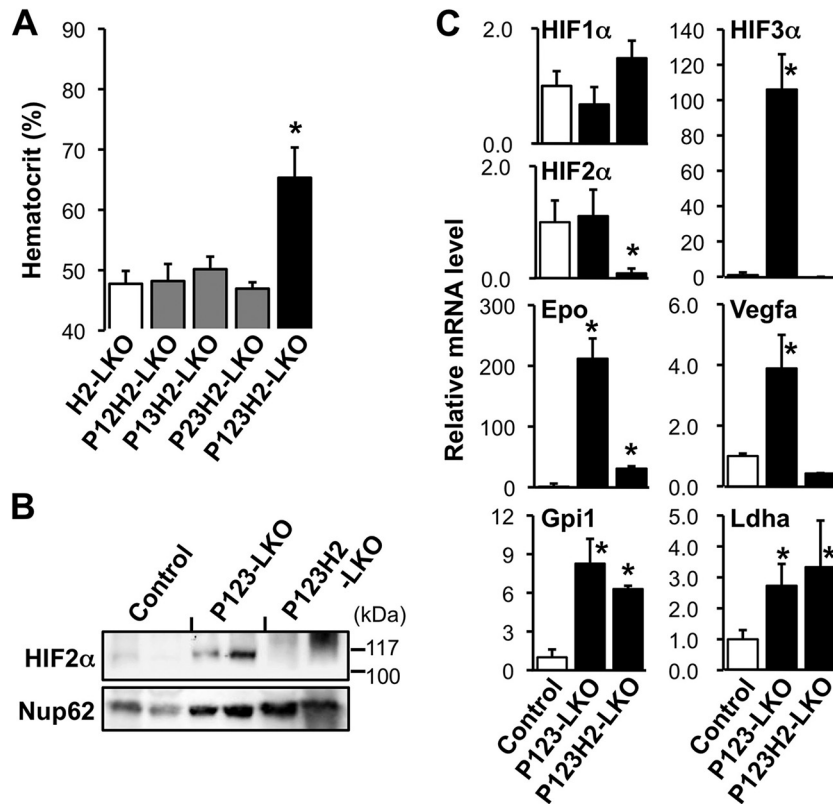


FIG 4 HIF2 α is essential for the development of polycythemia in liver-specific PHD knockout mice. (A) Hematocrit levels in PHD and HIF2 α mutant mice at 8 to 16 weeks of age are shown as the means and SD ($n = 3$ to 5 for each group). *, $P < 0.01$ compared with control H2-LKO mice. (B) Immunoblots for HIF2 α in nuclear extracts from the livers of PHD and HIF2 α mutant mice at 4 to 10 weeks of age. Nup62 was used as an internal control. The data from 2 independent samples are shown for each genotype group. (C) mRNA expression levels in the livers of PHD and HIF2 α mutant mice were analyzed by RT-qPCR. β -Actin was used as an internal control for RT-qPCR. The expression levels of control mice were set as 1.0. The data are means and SD ($n = 3$ for each group). *, $P < 0.01$ compared with the control mice (white bar). Note that the overexpression of *Epo* mRNA in P123-LKO livers is attenuated by the loss of HIF2 α (P123H2-LKO).

and severe steatosis in P123-LKO mice were also rescued by HIF2 α deficiency, the hyperactivation of HIF2 α most likely contributes to these two phenotypes.

Although the immunoblotting (Fig. 4B) and RT-qPCR (Fig. 4C) data showed that HIF2 α expression was almost absent in hepatocytes of P123H2-LKO mice, the overexpression of the *Epo* gene and the polycythemia observed in P123-LKO mice were not completely rescued by HIF2 α deletion (Fig. 4A and C). Thus, we suggest that HIF1 α accumulation may contribute to the overexpression of *Epo* mRNA in the triple-knockout livers. The overexpression of the *Vegfa* gene was also rescued by HIF2 α deficiency in P123-LKO livers, while mRNA expression of the glycolytic enzymes (*Gpi1* and *Ldha*) was present at high levels regardless of the HIF2 α status (Fig. 4C). These gene expression profiles indicated that HIF2 α , rather than HIF1 α , upregulates the transcription of the *Epo* and *Vegfa* genes in hepatocytes under hypoxic conditions, whereas the glycolytic genes are primarily regulated by HIF1 α . Interestingly, the loss of all three PHD isoforms in hepatocytes strongly (100-fold) increased the HIF3 α mRNA level; this overexpression was also rescued by HIF2 α deficiency (Fig. 4C).

The *Epo* gene hepatic enhancer is required for *Epo* overproduction in PHD-deficient livers. We previously reported that an enhancer sequence that contains a HIF-binding sequence (hypoxia-responsive element [HRE]) and that is proximally downstream of the *Epo* gene transcriptional endpoint is necessary and suffi-

cient for the expression of the *Epo* gene in the liver (12). A mouse line (Δ *EpoHE*) lacking 500 bp of the hepatic enhancer for the *Epo* gene (*EpoHE*) presents with fetal anemia due to a loss of *Epo* production by the fetal liver, which is the major *Epo*-producing site in fetuses. In contrast, adult Δ *EpoHE* mice exhibit normal erythropoiesis because adult mice produce most of their *Epo* in the kidneys, where *Epo* expression is independent of *EpoHE* (12). Δ *EpoHE* mice were crossed with liver-specific PHD-deficient mice to determine whether *EpoHE* is a *cis*-regulatory element for the hepatic *Epo* expression induced by the PHD-HIF2 α signaling cascade.

The polycythemia observed in PHD-mutant mice was completely rescued by the loss of *EpoHE* (Fig. 5A), demonstrating the essential function of *EpoHE* in hepatic *Epo* induction by the PHD-HIF2 α hypoxia signaling cascade. Indeed, *Epo* expression in the livers of double (Fig. 5B)- and triple (Fig. 5C)-knockout mice with the *EpoHE* deficiency fell within the normal range. These data clearly showed that *EpoHE* is essential for the induction of hypoxia-inducible *Epo* expression by the PHD-HIF2 α signaling cascade.

HIF3 α mRNA levels were unaffected by the loss of *EpoHE* (Fig. 5C), indicating that *EpoHE* functions solely in *Epo* gene regulation. In addition, *EpoHE* loss did not rescue the severe steatosis (Fig. 5D), the elevated glycogen deposition (Fig. 5E), or the downregulation of *Scd1* expression (Fig. 5C) observed in PHD123-LKO livers. Additionally, the lethality of P123-LKO mice was not res-

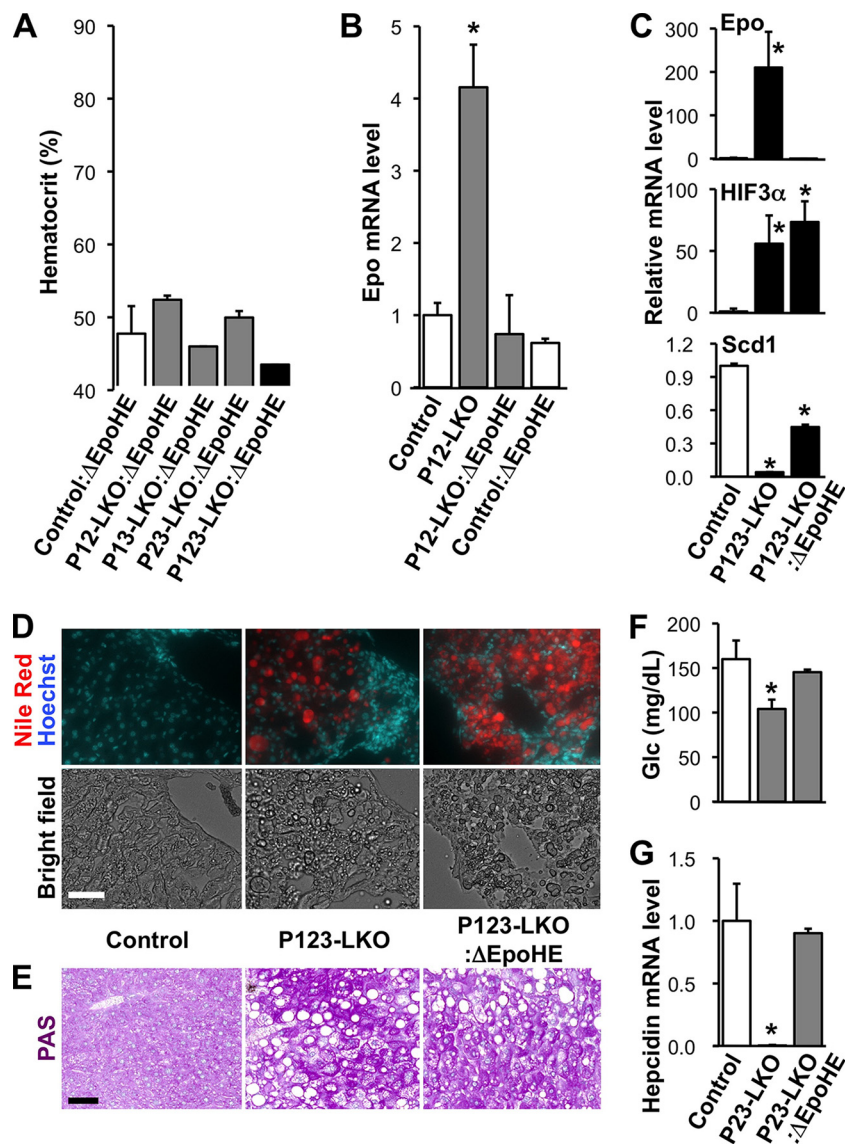


FIG 5 The hepatic enhancer for the *Epo* gene (*EpoHE*) is necessary for the development of polycythemia in PHD mutant mice. (A) Hematocrit levels in the PHD and *EpoHE* mutant mice at 6 to 15 weeks of age. The data are means \pm SD ($n = 3$ for each group). (B) *Epo* mRNA expression levels in the livers of PHD and *EpoHE* mutant mice at 8 to 15 weeks of age. The expression levels of control mice were set as 1.0 after normalization to β -actin mRNA levels. The data are means \pm SD ($n = 3$ for each group). *, $P < 0.01$ compared with the levels in the control mice (left bar). (C) Expression levels of *Epo*, *HIF3 α* , and *Scd1* mRNAs in the livers of PHD and *EpoHE* mutant mice were analyzed by RT-qPCR. β -Actin was used as an internal control. The expression levels of control mice were set as 1.0. The data are means \pm SD ($n = 3$ or 4 for each group). Note that the overexpression of *Epo* mRNA in P123-LKO livers is completely suppressed by the loss of *EpoHE* (P123-LKO: Δ *EpoHE*). (D) Lipid droplets in the livers of PHD mutant mice at 7 weeks after birth were stained with Nile red (red). Hoechst 33342 was used for nuclear staining (blue). (E) Glycogen deposition in the livers of the PHD and *EpoHE* mutant mice at 8 weeks of age was detected by PAS staining (purple). (F) Glucose concentrations were measured in peripheral blood from the PHD and *EpoHE* mutant mice at 8 weeks of age. The data are means \pm SD ($n = 3$ for each group). (G) Hepcidin mRNA expression in the livers of PHD and *EpoHE* mutant mice was analyzed by RT-qPCR. β -Actin was used as an internal control. The expression levels in the control mice were set as 1.0. The data are means \pm SD ($n = 3$ or 4 for each group). Bars, 50 μ m (D and E). *, $P < 0.01$ compared with the control mice (C, F, and G).

cued by the *EpoHE* deletion. These findings suggested that hepatic steatosis and glycogen deposition are caused by the hyperactivation of HIFs but are not related to *Epo* overproduction or to polycythemia.

The peripheral blood glucose concentration in the PHD double-knockout mice was decreased compared with that in the control mice, and these reduced blood glucose levels were restored by *EpoHE* loss (Fig. 5F). This observation indicates that the decreased blood glucose levels are caused by polycythemia but are not re-

lated to HIF hyperactivation in liver-specific PHD-deficient mice because erythrocytes actively uptake and utilize blood glucose for energy production (48).

Iron homeostasis is mainly regulated by hepcidin, which inhibits iron usage for erythropoiesis, and hepcidin secretion from livers is downregulated in mice actively undergoing erythropoiesis (49). Hepcidin mRNA levels were dramatically decreased in livers from polycythemic PHD double-knockout mice compared with control mouse livers (Fig. 5G). In nonpolycythemic P23-LKO:

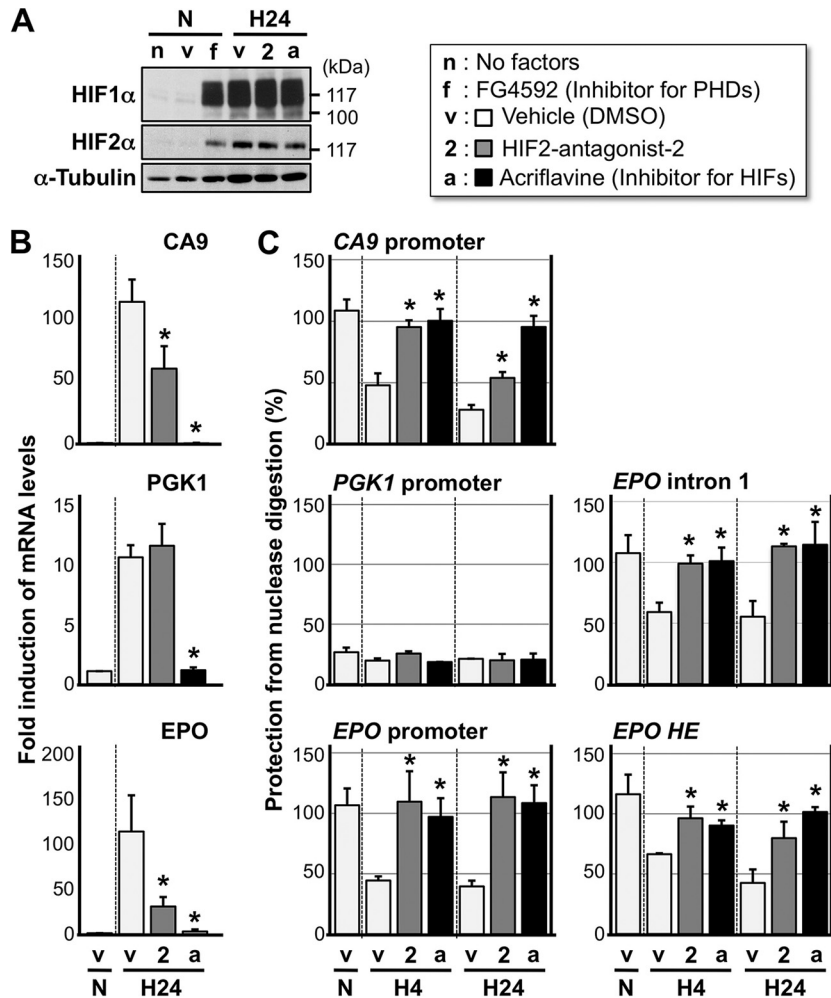


FIG 6 A HIF2 α -specific inhibitor blocks the dissociation of nucleosome structures in the *EPO* gene under hypoxic conditions in human hepatocytes. (A) Immunoblots for HIF1 α and HIF2 α in Hep3B human hepatoma cells under normoxic (N) and hypoxic (H24 [1% oxygen for 24 h]) conditions. α -Tubulin was used as an internal control. Both HIF1 α and HIF2 α proteins accumulated in cells treated with a PHD inhibitor (FG4592) or in cells under hypoxic stimulation, and the protein levels were not altered by supplementation with HIF inhibitors (HIF2 antagonist 2 and acriflavine) under hypoxic conditions. (B) RT-qPCR of CA9, PGK1, and *EPO* mRNA expression in Hep3B cells cultured with vehicle (v) or HIF inhibitors (HIF2 antagonist 2 and acriflavine) under normoxic (N) or hypoxic (H24) conditions. β -Actin was used as an internal control for RT-qPCR. The expression levels under normal conditions with vehicle supplementation (v/N) were set as 1.0. The data are means and SD from 4 independent experiments. *, $P < 0.01$ compared with hypoxic samples supplemented with vehicle (v/H24). A HIF2 α -specific inhibitor (HIF2 antagonist 2) strongly suppressed hypoxic induction of *EPO* expression but did not affect the expression of *PGK1*. (C) Nucleosome occupancy of chromatin loci for the *CA9*, *PGK1*, and *EPO* genes was detected via nuclease accessibility in Hep3B cells cultured with vehicle (v) or HIF inhibitors (HIF2 antagonist 2 and acriflavine) under normoxic (N) or hypoxic (H4 [1% oxygen for 4 h] and H24 [1% oxygen for 24 h]) conditions. The indicated loci were detected by qPCR of nuclease-digested and undigested chromatin from Hep3B cells, and the amount found in undigested samples was set as 100%. The values are means and SD from 4 independent experiments. *, $P < 0.01$ compared with vehicle-treated samples (v) under each condition. Nuclease accessibility was high in the *PGK1* promoter region in all samples, indicating that this region is free from nucleosome structure under both normoxic and hypoxic conditions. The promoter, gene body (intron 1), and hepatic enhancer (*HE*) of the *EPO* gene and the *CA9* promoter region were well protected from nuclease digestion under normal conditions (v/N), and nuclease accessibility increased under hypoxic conditions. Note that the formation of the nucleosome-free region under hypoxic conditions was repressed by HIF inhibitors.

Δ EpoHE mice, hepcidin mRNA levels were normal (Fig. 5G). This result is consistent with a previous report that hepcidin expression is suppressed by active erythropoiesis but not by direct effects of Epo or HIFs (49).

HIF2 α is involved in nucleosome reorganization around the *EPO* gene in hepatocytes. Next, we analyzed the chromatin state of HIF target genes in human hepatoma Hep3B cells that express both HIF1 α and HIF2 α proteins in a hypoxia-inducible manner to investigate how HIF2 α activates transcription of the *EPO* gene in hepatocytes (Fig. 6A). In Hep3B cells, the expression of *EPO*

and the expression of the well-known HIF targets carbonic anhydrase 9 (CA9) and phosphoglycerate kinase 1 (PGK1) were strongly induced by hypoxia (1% oxygen for 24 h) (Fig. 6B). HIF2 α -specific inhibitor (HIF2 antagonist 2) (38) significantly decreased CA9 and *EPO* mRNA levels under hypoxic conditions, while the *PGK1* mRNA level was not affected (Fig. 6B). In contrast, acriflavine, which is an inhibitor for both HIF1 α and HIF2 α (50), suppressed the expression of all three HIF target genes (Fig. 6B). Because both compounds inhibit the heterodimerization of HIF α and - β subunits (38, 50), these results corroborate the re-

sults from P123H2-LKO mice, which suggested that *Epo* gene expression is primarily activated by HIF2 α and not by HIF1 α . Indeed, previous studies utilizing RNA interference demonstrated that HIF2 α -specific suppression, but not HIF1 α -specific suppression, dramatically reduces hypoxia-inducible *Epo* gene expression in *Epo*-producing hepatic cell lines (51, 52).

Next, we used a qPCR-based technique to analyze the changes in nuclease accessibility (sensitivity) to chromatin DNA in Hep3B cells that were exposed to hypoxia (39). Nuclease-sensitive sites are often an indication of transcriptionally active genomic regions, in which histones have dissociated from nucleosomes to open the chromatin for transcription factor binding. Under normal conditions, chromatin DNA was highly protected from nuclease digestion in the promoter, gene body (intron 1) and enhancer (*HE*, a hepatic enhancer downstream of the transcription end site) regions of the *EPO* gene (Fig. 6C). Hypoxic stimuli (1% O₂ for 4 or 24 h) increased the accessibility of the *EPO* gene locus (Fig. 6C). The *CA9* promoter was similarly affected by hypoxia, whereas the *PGK1* promoter remained sensitive to nucleases (Fig. 6C). These data demonstrated that the *CA9* and *EPO* genes in Hep3B cells are covered by histones under normal conditions and that the nucleosome structure is disassembled under hypoxic conditions, facilitating active transcription. The *PGK1* promoter is always nucleosome free to enable interactions with transcription factors and to achieve the high level of gene expression.

HIF2 antagonist 2 blocked the hypoxia-induced formation of open chromatin structures in the *EPO* and *CA9* gene loci (gray bars in Fig. 6C). Acriflavine treatment also caused these chromatin regions to become insensitive to nuclease digestion under both 4- and 24-hour hypoxic conditions (Fig. 6C). These data corroborate our gene expression data showing that both inhibitors suppress *EPO* and *CA9* gene expression. These inhibitors did not have any effect on the constitutively open chromatin locus of the *PGK1* gene (Fig. 6C). Thus, we concluded that HIF2 α is required for the formation of open chromatin structures in the *EPO* gene locus to induce active transcription under hypoxic conditions in hepatocytes.

HIF3 α is induced by hypoxia and attenuates HIF2 α -mediated *EPO* transcription in hepatocytes. Because we discovered that HIF3 α mRNA expression, similar to *Epo* mRNA expression, is strongly induced by the loss of PHD isoforms in livers in a HIF2 α -dependent manner (Fig. 4C and 5C), the role of HIF3 α function in hepatic *Epo* gene regulation was further analyzed. The *Hif3a* gene in mice mainly generates two splicing isoforms, IPAS and NEPAS, in addition to full-length HIF3 α (18, 19). NEPAS is exclusively expressed at embryonic and neonatal stages, whereas IPAS expression is induced by hypoxia in the hearts, lungs, and muscles of adult mice (18). We determined the expression of IPAS-specific transcripts in P123-LKO livers and found that IPAS expression was only weakly induced by PHD deficiency, compared to the strong induction of full-length HIF3 α expression caused by PHD deficiency (Fig. 7A). HIF3 α mRNA expression in the mouse liver was also stimulated by exposure to hypoxic stress (6% oxygen for 48 h) (Fig. 7B). These data indicated that full-length HIF3 α expression is induced by hypoxia through the PHD-HIF pathway in the liver.

We analyzed the activity of *EpoHE* in Hep3B cells that overexpress human full-length HIF3 α to investigate the function of HIF3 α in *EpoHE*-mediated gene regulation. We detected the nucleus-specific localization of overexpressed HIF3 α , which was

conjugated with HaloTag, via staining with a cell-permeable HaloTag ligand in living Hep3B cells (Fig. 7C). The expression of a luciferase reporter driven by *EpoHE* was significantly increased by HIF2 α overexpression, and this induction was suppressed by HIF3 α in a dose-dependent manner (Fig. 7D). These data indicated the functional importance of HIF3 α in hypoxia-inducible *Epo* gene regulation. After the removal of the HaloTag by TEV protease, the Western blot data confirmed that the overexpressed HIF3 α was the 80-kDa full-length HIF3 α (HIF3 α 1) (20) and that HIF2 α protein levels in the constitutively active HIF2 α mutant were not affected by HIF3 α overexpression (Fig. 7E). Taken together, our results suggest that HIF3 α expression in the liver is induced by hypoxia to limit HIF2 α -mediated *Epo* overproduction (Fig. 7F).

DISCUSSION

The REP cells in the renal tubular interstitium are the site of the majority of the *Epo* production in adult animals (9, 13); the contribution of the liver, which is the other *Epo*-producing organ, to adult erythropoiesis is extremely small (12). Therefore, damage to the REP cells in chronic kidney diseases causes renal anemia even in patients with an intact liver. Strategies that inhibit PHDs by using small molecules or RNA interference have been investigated in an attempt to develop a treatment that would enhance hepatic *Epo* production in patients with renal anemia (14, 15, 53). However, PHD inhibition induces several genes, including oncogenes, steatosis-related genes, and the *Epo* gene, via the activation of both HIF1 α and HIF2 α (54). In the present study, we investigated the *in vivo* regulatory mechanisms of hepatic *Epo* production to identify therapeutic targets that exclusively stimulate *Epo* production.

The liver-specific knockouts of each PHD isoform exhibited no apparent phenotype, indicating that PHDs have redundant functions in regulating the hypoxia-response pathway (28, 29). The loss of all three genes for PHD isoforms in the liver resulted in *Epo* overproduction and polycythemia via the upregulation of both HIF1 α and HIF2 α . However, the strong and constitutive activation of HIFs caused steatosis of the liver and the expression of other HIF target genes (29), and the mice died approximately 10 weeks after birth. We speculate that the development of fatty livers in the P123-LKO mice is related to the HIF-mediated inactivation of mitochondria, which are the organelles that utilize intracellular fatty acids (55). We also showed that these phenotypes depended on neither polycythemia nor *Epo* overproduction by examining P123-LKO mice lacking *EpoHE*, which exhibited HIF overexpression and lethal steatosis but not *Epo* overproduction. The data from the P123-LKO mice suggested that PHD inhibition is responsible for the induction of hepatic *Epo* production, although universal PHD inhibitors not only induce *Epo* production but also produce dangerous side effects.

Our data demonstrated that the disruption of two PHD isoforms was sufficient to activate erythropoiesis via *Epo* gene induction in hepatocytes, without the severe steatosis that was observed in the P123-LKO livers. Surprisingly, the mice lacking two PHD isoforms in any combination exhibited similar polycythemia phenotypes, suggesting that no significant functional differences in hepatic *Epo* gene regulation exist among the three PHD isoforms. However, we believe that these isoforms may have specific roles in other parts of the hypoxia response pathway because these isoforms have different affinities for HIFs, which differ in protein structure outside their catalytic domains (56).

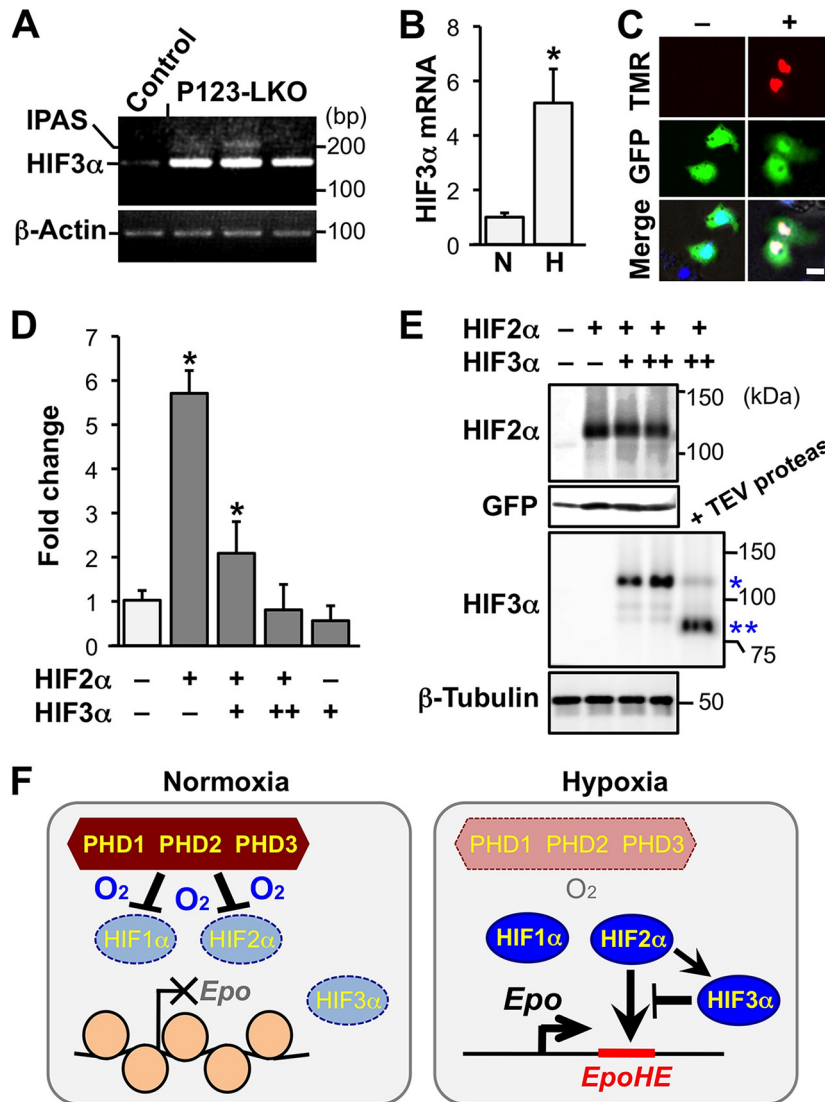


FIG 7 Involvement of HIF3α in the hypoxic regulation of *Epo* expression. (A) Detection of the IPAS-specific splicing variant of the *Hif3a* gene by RT-PCR in livers of P123-LKO and control mice. β-Actin was used as an internal control. The 207-bp PCR amplicon derived from the IPAS splicing variant was undetected or detected at extremely low levels by agarose gel electrophoresis of PCR samples compared with the 151-bp amplicon derived from HIF3α-specific splicing variants. (B) Expression levels of HIF3α mRNA in livers of wild-type mice exposed to normal (N) or hypoxic (6% oxygen) air for 48 h were measured by RT-qPCR. The expression levels of normoxic samples were set as 1.0. The data are means and SD from 3 independent mice for each condition. *, $P < 0.01$ compared with normoxic samples. (C) Overexpression of HIF3α in the nuclei of living Hep3B cells. Hep3B cells were transfected with a control plasmid expressing GFP (pEGFP-N1 [green]) with (+) or without (–) the HaloTag-HIF3α expression plasmid. HaloTag-HIF3α was detected with a HaloTag-TMR direct ligand (red) in Hoechst 33342-stained nuclei (blue [merged images in lower panels]). Bar, 10 μm. (D) Luciferase reporter analysis of *EpoHE* in Hep3B cells overexpressing HIF2α and/or HIF3α. The HIF2α expression plasmid and/or the HIF3α expression plasmid (+, 300 ng/well; ++, 600 ng/well) were cotransfected with the *EpoHE*-reporter plasmid. The relative luciferase activity of the control samples (white bar) was set as 1.0 after normalization with internal controls. The values are means and SD from 4 independent experiments. *, $P < 0.01$ compared with control samples. (E) Expression of HIF2α and HIF3α in cells that were used for the *EpoHE* reporter assay was confirmed by immunoblotting. The constitutively active HIF2α mutant was detected using anti-HIF2α antibody in whole-cell lysates from the cells used for panel D. Dose-dependent expression of HaloTag-HIF3α (*) was detected using anti-HIF3α antibody. HaloTag-cleaved HIF3α (**) was also detected in a TEV protease-digested sample. GFP and β-tubulin were used as loading controls. (F) A model of hypoxia-inducible *Epo* gene regulation in hepatocytes. Under normoxic conditions, PHDs suppress the activities of HIF1α and HIF2α via protein degradation, and nucleosome structures on the chromatin silence the *Epo* gene. In hypoxic cells, HIF1α and HIF2α are activated by the inactivation of PHDs. HIF2α induces *Epo* transcription via the disassembly of the nucleosome structures. The HIF2α-mediated *Epo* induction is attenuated by HIF3α, which is also induced by HIF2α.

Although the level of *Epo* mRNA expression in the double-knockout livers was approximately 10-fold higher than that of the control mice, their plasma *Epo* concentrations were within the normal range. *Epo* is internalized and degraded in *EpoR*-expressing erythroid cells after the transduction of erythropoietic signaling (42). Therefore, our data suggest that most of the *Epo* pro-

duced by the double-knockout livers is absorbed by erythroblastic cells expressing *EpoR* at a high level, which increase more than 10-fold in number in the spleens of double-knockout mice. The plasma concentration of thrombopoietin (Tpo) depends on the quantity of c-mpl (Tpo receptor)-expressing platelets in peripheral blood (so-called “sponge theory”) (57). Thus, we propose a

sponge theory for Epo, in which plasma Epo levels are related to the number of erythroblastic cells in spleens.

Polycythemia in the liver-specific PHD knockout mice was rescued by liver-specific HIF2 α deficiency. This result is comparable to that of a previous study, which showed that HIF2 α is required for the development of polycythemia in liver-specific knockout of pVHL, which is an E3 ligase of HIF α subunits (26). These findings clearly indicate that HIF2 α , rather than HIF1 α , is the major trans-activator for *Epo* gene expression. Additionally, we showed that polycythemia was completely rescued by the loss of *EpoHE*. Taken together, our data suggest that HIF2 α binding to *EpoHE*, which contains an HRE, triggers Epo production in the liver and that *EpoHE* is the only *cis*-regulatory element for hypoxia-inducible *Epo* gene expression in hepatocytes (12). Patients with hepatocellular carcinoma occasionally present both polycythemia and Epo overproduction (58). In these cases, PHDs or other HIF suppression systems might be disrupted in the malignant cells. Recently, hereditary polycythemia has been linked to mutations in the PHD2 and HIF2 α genes (59–63), indicating that a PHD-HIF2 α pathway regulates *EPO* gene expression in humans.

Chromatin analyses demonstrated that the genomic DNA around the *Epo* gene, including the promoter and *EpoHE*, associates with histones under normal conditions. Under hypoxic conditions, the nucleosome structure is disassembled in a HIF2 α -dependent manner to activate *Epo* transcription (Fig. 7F). Chromatin remodeling factors, such as BRG1, BRM, and SWI/SNF, are implicated in HIF-mediated transcriptional activation (64, 65). Therefore, these factors may be recruited to the *Epo* gene locus by HIF2 α , which binds to *EpoHE* in hypoxic cells.

We found that HIF3 α expression is strongly induced by PHD deficiency in the liver and that this induction requires HIF2 α . Some reports have shown that HIF1 α , but not HIF2 α , activates *Hif3a* gene expression via binding to its HREs (20, 66). However, we suggest that hypoxia-inducible *Hif3a* gene expression in normal mouse liver is regulated primarily by HIF2 α . Because splicing variants of HIF3 α exist, the function of HIF3 α in hypoxia-inducible gene regulation is controversial (18, 19, 67, 68). In the present study, we demonstrated that full-length HIF3 α is primarily induced in hypoxic livers and that this expression attenuates transcriptional activation driven by the HIF2 α -*EpoHE* axis (Fig. 7F). Since HIF2 α deletion did not completely reduce the overexpression of the *Epo* gene, which was observed in P123-LKO mice, we suggested that HIF1 α secondly contributed to the overexpression of *Epo* mRNA. Alternatively, loss of *Hif3a* gene induction by HIF2 α deletion may be related to the incomplete reduction of the *Epo* overexpression in the quadruple-knockout livers.

As shown by the dramatic induction of hepatic Epo production following the genetic or pharmacological inactivation of PHDs, PHDs actively inhibit the function of HIFs, even in renal-anemia patients. Therefore, it is plausible to inhibit PHDs in the liver to treat renal anemia. However, mouse genetic studies have demonstrated that the universal inhibition of PHDs strongly induces the activation of HIFs and causes side effects, including steatosis (54, 69). To avoid severe side effects caused by robust inhibition of PHDs, one reasonable strategy suggested by this study is to establish mechanisms to specifically induce HIF2 α -*EpoHE* binding. Because chemical inhibitors usually only partly suppress enzymatic activity and their efficacy can be regulated by dosage, chemical compounds that inhibit PHDs have been tested in clinical trials for renal anemia (14). Additionally, it was recently reported that

treating mice with a PHD inhibitor (FG4497) improves metabolic dysfunction, including hepatic steatosis (70). This evidence indicates that renal anemia can be treated with PHD inhibitors.

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Y.T. and N.S. designed the study. Y.T., H.S., I.H., X.P., T.S., N.T., and N.S. performed the experiments. K.T., G.-H.F., T.D., T.M., M.Y., and N.S. provided the gene-modified mouse lines. T.T., S.-I.K., and T.M. provided the chemical compounds. Y.T., H.S., and N.S. analyzed the data and constructed the figures. Y.T. and N.S. wrote the manuscript. M.I., T.M., M.Y., and N.S. developed the project.

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